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(57) Abstract

The present invention provides magnetic resonance imaging (MRI) contrast agents useful for detecting thrombus. The MRI contrast agents comprise a chelator capable of complexing a paramagnetic metal which chelator is coupled to a chemical compound capable of binding to an integrin. The MRI contrast agent can additionally contain the paramagnetic metal which complexes with the chelator. Compositions containing these MRI agents and a physiologically acceptable carrier, as well as the use of such agents to detect thrombus in a subject, are also provided by the present invention.

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MAGNETIC RESONANCE IMAGING OF THROMBI

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to the field of diagnostic imaging and, more specifically, to magnetic resonance imaging for thrombi.

BACKGROUND INFORMATION

Cardiovascular disease is a devastating problem in the United States and worldwide. Diagnosis of thrombus is critical to the detection and management of cardiovascular disease. Major indications for thrombus imaging include coronary artery occlusion, deep vein thrombus, pulmonary emboli, stroke, and other such maladies. The U.S. and European patient populations with a need for diagnostic imaging procedures to be performed related to these maladies number in the tens of millions per year. Several previous methods attempted to provide effective diagnostic imaging of thrombi, but each of these methods has undesirable aspects.

Magnetic resonance imaging for diagnostic purposes has previously been used. However, such methods only detected thrombi as ambiguous negative signal areas, which can be misdiagnosed as anatomical irregularities. Although new software is improving the thrombus-imaging capability of MRI, MRI agents are currently unavailable that can localize a thrombus by rendering a positive image. Thus, a need exists for a MRI contrast agent that provides a positive image of a thrombus.

This need has been partially satisfied by MRI agents that are linked to antibodies that bind to thrombi. When such agents are administered to a patient,

the agents home to the thrombi, resulting in positive imaging of the thrombus. However, such agents have other serious disadvantages. For example, antibody-linked agents are not stable in a patient's circulatory system and consequently degrade. Moreover, when administered to the patient, such agents require long periods of time to clear from the patient's circulation, and may cause potential complications of an immune response by the patient to a foreign antibody. Most significantly, such agents have relatively low or nonspecific affinities to thrombi. Thus, the need remains for a highly sensitive method of positive imaging of thrombus that is stable and has rapid clearance.

Another previous method for detecting thrombus was to use radiolabeled small synthetic peptides that specifically home to thrombi. Compared to antibody linked contrast agents, such peptides have shorter clearance times, reduced cross-reactivity, and are more selective for binding to thrombi. Previously, however, such peptides have only been used in conjunction with radioimmunoscintigraphy, which is a technique that uses a γ-camera to detect radioactively labeled agents that are administered to patients. One study used a peptide labeled with ^{99m}Tc to detect deep venous thrombi in an animal model and in a preliminary human trial.

The drawback of this technique is that the use of ^{99m}Tc as the label for the probe still require the use of a γ-camera for imaging with its inherently poor resolution and slow scan times. This particular peptide does not have sufficiently high binding affinity for the receptors on the platelets to yield thrombus signal to background ratios greater than two. In addition, the large doses of ^{99m}Tc required by this agent expose the patient to significant levels of radiation (10-22 mCi). Moreover, injection of such agents cause discomfort and

present a risk to the patient. Although the use of radiolabeled peptides in radioimmunoscintigraphy presented certain advantages, the need remains for a method of providing positive diagnostic imaging of thrombi without the poor resolution and radiation exposure associated with the technique.

Thus, a need still exists for a magnetic resonance imaging agent which provides a positive image of a thrombus, which uses small molecules having rapid clearance times and which have high affinity for thrombi. The present invention satisfies these needs and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides MRI contrast agents useful for detecting thrombus. The MRI contrast agents comprise a chelator and a chemical compound capable of binding to an integrin. The MRI contrast agent can, though need not, additionally contain a paramagnetic metal. The chelator should be capable of complexing the metal and, if present, does complex the metal. Additionally, the chelator is also coupled to the integrin-binding chemical compound. The integrin-binding compound can be any variety of compounds which bind the RGD site of an integrin and, therefor, have cell attachment activity. The compounds can be linear or cyclic and, generally, will be X-G-D-containing (SEQ ID NO. 23), wherein X is a positively charged amino acid or a hydrophobic amino acid.

In a particular embodiment of the invention, the integrin-binding compound has the general structure $Y-X_1-X_2-G-D-X_3-X_4-Z$ (SEQ ID NO. 1). Within this

formula, Y is an amino-terminal group, X_1 is zero to five amino acids, X_2 is a hydrophobic amino acid or a positively charged amino acid, X_3 is a hydrophobic amino acid, an amino acid that provides an ionic interaction with an integrin receptor, or an amino acid that provides a hydrogen bond with an integrin receptor, X_4 is zero to five amino acids, and Z is a carboxy-terminal group. The compounds are cyclized through a bridge between two amino acids, excepting the G and D amino acids in the formation of the bridge.

The present invention also provides a composition useful as a medicament comprising an MRI contrast agent and a physiologically acceptable carrier.

The present invention further provides a method for detecting thrombus in a subject by administering to a subject an effective amount of an MRI contrast agent, and when the MRI agent homes to a thrombus, detecting the thrombus using magnetic resonance imaging.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides MRI contrast agents useful for detecting thrombus. The subject MRI contrast agents comprise at least a chelator coupled to and an integrin-binding chemical compound. The agents can further comprise a paramagnetic metal. Each of these components of the MRI agents of the present invention will be discussed in turn below.

CHELATORS

In the present invention the chelator is coupled to the integrin-binding chemical compound. It is capable of complexing a paramagnetic metal, and when present, does complex the metal. As used herein, a "chelator" is a molecule with one or more polar groups that act as a ligand for, and can complex with, the metal. As used herein, the phrase "capable of complexing a paramagnetic metal" means the chelator contains chemical groups which have the ability to complex a paramagnetic metal by noncovalent forces. As used herein, the phrase chelator complexes the metal" means an aggregate of the chelator and metal ion held together by noncovalent forces.

Chelators for metals are well known in the art, and include acids with methylene phosphonic acid groups, methylene carbohydroxamic acid groups, carboxyethylidene groups, or carboxymethylene groups. Examples of chelators include, but are not limited to, diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane (DOTA), ethylenediaminetetraacetic acid (EDTA) and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA). In a preferred embodiment of the present invention, the chelator of the MRI agent is DTPA.

In the present invention, the chelator of the MRI contrast agent is coupled to the integrin-binding chemical compound. The two are preferably coupled through the Y or Z substituents or through an amino acid present in X_1 or X_4 as described in further detail below. As used herein, the term "coupled" broadly includes any attachment of the chelator to the compound. The attachment can be covalent or noncovalent, although more

usually covalent, as with for example, conjugation using DTPA, described in Example III. The coupling may be direct or, more commonly by indirect means of using a coupling or cross-linking reagent. Any one of several known intermolecular cross-linking reagents can be used, including N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) or N-N'-(1,3-phenylene)bismalemide (both highly specific for sulfhydryls, forming irreversible linkages); N-N'-ethylene-bis-(iodoacetamide) or other such reagents having 6 and 11 carbon methylene bridges (relatively specific for sulfhydryl groups); 1,5-difluoro-2,4-dinitrobenzene (forming irreversible linkages with amino and tyrosine groups); *p,p'*-difluoro-*m-m'*dinitrodiphenylsulfone (forming irreversible cross-linkages with amino and phenolic groups); dimethyladipimidate (specific for amino groups); phenyl-2-4-disulfonylchloride (reacting principally with amino groups); hexamethylenediisocyanate or diisothiocyanate, or azophenyl-*p*-diisocyanate (reacting principally with amino groups); glutaraldehyde (reacting with several different side chains); and bis-diazobenzidine (reacting primarily with tyrosine and histidine). Additional coupling or cross-linking reagents are known in the filed, as described in, for example, Means & Feeney, Chemical Modification of Proteins, Holden-Day, pp. 39-43 (1974).

A skilled artisan will readily appreciate that the chelator and the coupling method will be selected to preserve the paramagneticity and minimize the toxicity of the paramagnetic metal, while preserving the specificity of integrin binding. Such considerations are discussed in Tweedle et al., Magnetic Resonance Imaging, vol. 1 (Eds. Partain et al., W.B. Saunders Co., 2nd ed.) pp. 798-806, 1988.

INTEGRIN-BINDING COMPOUND

The integrin-compound used in the MRI agents of the present invention can be any small, synthetic compound which binds the RGD site of an integrin and, therefore, has cell attachment activity. Such small compounds have rapid clearance times, are sufficiently stable for diagnostic purposes, and have high affinity for integrins, making their application in MRI contrast agents particularly advantageous.

The compounds are X-G-D-containing (SEQ ID NO. 23) wherein X is a positively charged amino acid or hydrophobic amino acid. By "X-G-D-containing" (SEQ ID NO. 23) is meant any molecule which has at least the sequence X-G-D.

Compounds containing X-G-D, wherein X is a positively charged amino acid are known in the art. They are known to bind the RGD integrin site and to have cell attachment activity. Such compounds can be linear, such as those described in U.S. Patent Nos. 4,792,525, 4,578,079, 4,614,517 and 4,879,237. Alternatively, such compounds can be cyclized, such as those described in WO 89/05150.

In the present invention, the chemical compound enables the MRI contrast agent to home to integrins on the surface of activated platelets, thereby enhancing MRI visualization of the thrombus. Thus, a critical aspect of the present invention is the ability of compounds of the MRI contrast agent to bind to integrins and do so with sufficient affinity. The structure of the integrin-binding compounds can vary substantially as described above. What is important is that the compound bind the

RGD site of an integrin and have sufficient affinity for one or more integrins as to be advantageous in their application to magnetic resonance imaging. A dose to allow a compound to bind with an affinity in the nanomolar range, meaning a range submicromolar, is of the appropriate affinity for MRI. Preferably the affinity is 1 to 10 nanomolar. Affinities of integrin-binding compounds can be routinely determined by methods well known in the art and by such methods as described in Example II.

In one embodiment of the invention, the integrin-binding compounds have the general structure Y-X_1-X_2-G-D-X_3-X_4-Z (SEQ ID NO. 1). Within this genus, Y is an amino-terminal group, X_1 is zero to five amino acids, X_2 is a hydrophobic amino acid or a positively charged amino acid, X_3 is a hydrophobic amino acid, an amino acid that provides an ionic interaction with an integrin receptor, or an amino acid that provides a hydrogen bond with an integrin receptor, X_4 is zero to five amino acids, and Z is a carboxy-terminal group. The compounds are cyclized through a bridge between two amino acids, excepting the G and D amino acids in the formation of the bridge.

As used herein the term "amino acid" is meant in its broadest sense to include naturally occurring proteogenic amino acids, D-amino acids and imino acids, as well as non-naturally occurring or chemically modified amino acids and imino acids and amino acid mimics. The term "proteogenic" indicates that the amino acid can be incorporated into a protein in a cell through well known metabolic pathways.

In view of this broad definition of an amino acid, one skilled in the art would know that a reference herein to an amino acid, unless specifically indicated otherwise, includes, for example, naturally occurring proteogenic L-amino acids and D-amino acids. The choice of including an L- or a D-amino acid depends, in part, on the desired characteristics of the compound of the MRI contrast agent. For example, the incorporation of one or more D-amino acids can confer increased stability on the MRI contrast agent *in vivo* and can allow the agent to remain in the body for an extended period of time.

A skilled artisan would also know that the term "amino acid" also encompasses chemically modified amino acids such as amino acid analogs, naturally occurring non-proteogenic amino acids such as norleucine and chemically synthesized compounds having properties known in the art to be characteristic of an amino acid. The one-letter and three-letter abbreviations for amino acids and derivatives thereof used herein are as follows:

One-letter	Three-letter	Amino acid and analogs thereof
	Adp	γ-aminoadipic acid
A	Ala	alanine
	βAla	β-alanine
	α-Aba	α-aminoisobutyric acid
R	Arg	arginine
N	Asn	asparagine
D	Asp	aspartic acid
	Cha	cyclohexylalanine
	Chg	cyclohexylglycine
	Cit	citrulline
	Dic	decahyroisoquinoline-3-carboxylic acid
C	Cys	cysteine
Q	Gln	glutamine

E	Glu	glutamic acid
G	Gly	glycine
	t-BuG	*tert*-butylglycine
H	His	histidine
	homoPhe	homophenylalanine
	homoArg	homoarginine
	Idc	indoline-2-carboxylic acid
I	Ile	isoleucine
L	Leu	leucine
K	Lys	lysine
M	Met	methionine
	Mpa	β-mercaptopropionic acid
	Msa	β-[(methylsulfonyl)amino]alanine
	Mamb	*m*-(aminomethyl)benzoic acid
	1-Nal	β-(1-naphthyl)alanine-OH
	2-Nal	β-(2-naphthyl)alanine-OH
	Nap	naphthylalanine analogs
	Nle	norleucine
N-Me-R	N-Me-Arg	N-methylarginine
	Npg	neopentylglycine
	Nve	norvaline
	Oic	octahydroindole-2-carboxylic acid
	OMeTic	O-methyl-Tic
	O-*n*-butyl-Tyr	O-*n*-butyl-tyrosine
	O-*n*-hexyl-Tyr	O-*n*-hexyl tyrosine
	Orn	ornithine
	p-amino-Phe	paraaminophenylalanine
	Pas	6,6,-cyclophentamethylene-2-aminosuberic acid
	Pen	penicillamine
	Phg	phenyl-glycine
F	Phe	phenylalanine
	p-amino-Phe	paraaminophenylalanine
	p-Cl-Phe	parachlorophenylalanine

		p-iodo-Phe	paraiodophenylalanine
		p-nitro-Phe	paranitrophenylalanine
		Pmc	β,β-pentamethylenecysteine
		Pmp	β,β-pentamethylene-β-mercaptopropionic acid
	P	Pro	proline
		homoPro	homoproline
		dhPro	dehydroproline
		Psa	β-[(phenylsulfonyl)amino]alanine
	S	Ser	serine
		Tca	1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid
		tetAla	β-(1(2)H-tetrazol-5-yl)-alanine
		Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
		7-OMe-Tic	7-O-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
	T	Thr	threonine
		Tfsa	β-[(trifluoromethylsulfonyl)amino]alanine
	W	Trp	tryptophan
	Y	Tyr	tyrosine
	Y-OMe	Tyr-OMe	O-methyl-tyrosine
	Y-OEt	Tyr-OEt	O-ethyl-tyrosine
	Y-O-*n*-Bu	Tyr-O-*n*-Bu	O-*n*-butyl-tyrosine
	V	Val	valine

A "mimic" or "mimetic" of an amino acid means an amino acid analog or non-amino acid moiety that has the same or similar functional characteristic of a given amino acid. For instance, an amino acid mimic of a hydrophobic amino acid is one that is non-polar and retains hydrophobicity, generally by containing an

aliphatic functional group. As a further example, an arginine mimic can be an arginine analog containing a side chain with a positive charge at physiological pH, which is characteristic of the guanidinium side chain of arginine.

Amino acid mimics also encompass modifications to the chemical or peptide bonds and backbone. Such modifications can be made to the amino acid, amino acid derivative, non-amino acid moiety or a peptide on either side. What is critical is that such modifications mimic a peptide backbone with substantially the same spatial arrangement and distance that is typical for traditional peptide bonds and backbones. An example of such a modification is reducing a carbonyl of the amide peptide backbone to an amine. Several reagents are available and well known for reducing amides to amines, such as those disclosed in Wann et al., J. Org. Chem, 46:257 (1981) and Raucher et al., Tetrahedron Lett., 21:14061 (1980). An amino acid mimic is, therefor, an organic molecule that retains the similar amino acid pharmacophore groups as is present in the corresponding amino acid and which exhibits substantially the same spatial arrangements between functional groups.

Substituting amino acid mimics and non-naturally occurring amino acids for naturally occurring amino acids can enhance the overall activity or properties of an individual compound. These alterations to the compound of the MRI contrast agent can enhance the compound's stability to enzymatic breakdown.

Each of the substituents of the generic formula $Y-X_1-X_2-G-D-X_3-X_4-Z$ (SEQ ID NO. 1) will now be described in detail, first with reference to the core

structure, X_1-X_2-G-D-X_3-X_4 (SEQ ID NO. 2), followed by a detailed description of the terminal groups, Y and Z.

In one embodiment of the invention X_1 is zero to five amino acids, X_2 is a hydrophobic amino acid or a positively charged amino acid, X_3 is a hydrophobic amino acid, an amino acid that provides an ionic interaction with an integrin receptor, or an amino acid that provides a hydrogen bond with an integrin receptor, and X_4 is zero to five amino acids.

As used herein, the term "hydrophobic amino acid" is intended to include naturally-occurring hydrophobic amino acids, including Ala, Val, Ile, Leu, Phe, Tyr and Trp as well as non-naturally-occurring hydrophobic amino acids, including the D-form of the hydrophobic amino acids, amino acid derivatives and amino acid mimics, which are non-polar. Hydrophobic amino acid derivatives and mimetics useful in the invention can have a range of structural types and hydrophobicities. Examples of such include, but are not limited to, Nle, Nve, Cha, Nap, 2-Nal, Cit, Tyr derivatives, Phe derivatives and Trp derivatives. Examples of additional hydrophobic amino acids include Chg, *t*-BuG, Msa, Npg, Psa, Tfsa and 1-Nal. Examples of Tyr derivatives include Tyr-OMe, Tyr-OEt, O-*n*-hexyl-Tyr, O-*n*-Bu-Tyr, 3,5-diiodo-Tyr and the like. Examples of Phe derivatives include p-chloro-Phe, homoPhe, p-nitro-Phe, Phg, p-iodo-Phe, p-amino-Phe and the like. Tryptophan derivatives are, for example, Trp amino acid analogs with a substituted indole ring, substituted with, for example, one or more halogen atoms including iodo, chloro, fluoro, and bromo atom(s), and/or one or more alkyl groups, such as methyl, ethyl, and the like.

As used herein, the term "positively charged amino acid" refers to those amino acids which occur in nature, including Arg, Lys and His, and the D-form of these naturally-occurring amino acids as well as amino acid derivatives and amino acid mimics, which are positively charged. Examples of additional positively charged amino acids include, but are not limited to, Orn and homoArg.

As used herein, "an amino acid that provides an ionic interaction with an integrin receptor" means any amino acid which interacts with an integrin receptor by attractive forces, preferably through providing a negative charge, as would be provided by such amino acids as Asp, Glu, β-(1(2)H-tetrazol-5-yl)-alanine or analogs or mimics thereof such as the sulfonamide derivatives Msa, Psa, Tfsa or other such negatively charged amino acids or mimics thereof.

As used herein, "an amino acid that provides a hydrogen bond with an integrin receptor" means any amino acid that provides an attractive interaction between its covalently linked hydrogen atom and a neighboring atom or group of atoms in the integrin receptor such as, for example, Asn, Ser, Thr, or mimics or analogs thereof.

In one embodiment of the invention, X_2 and X_3 are both a hydrophobic amino acid and X_4 is one to five amino acids, at least one of which is a positively charged amino acid directly adjacent to X_3. In a more preferred embodiment, the hydrophobic amino acid of either X_2 or X_3 are, independently, Ala, *t*-BuG, Tic, Val, Ile, Leu, Phe, Tyr, Trp, a Tyr derivative, a Phe derivative, Nle, Nve, Cha, Nap, 2-Nal or Cit; and X_4 is at least one positively charged amino acid selected from

Arg, Lys, His, Orn or homoArg which amino acid is directly adjacent to X_3. In a more preferred embodiment, X_2 is Ala, Val, Ile, Leu, Phe, Tyr, Trp, Nve, Nle, Cha or Nap; X_3 is Phe, a Phe derivative, Tyr, a Tyr derivative or Trp; and X_4 contains at least an Arg amino acid residue directly adjacent to X_3. Even more preferably, X_2 is Ala, Leu or Nle and X_3 is Tyr-OMe. Preferred compounds falling within these formulae include (Nle)GD(Y-OMe)REK-NH_2 (SEQ ID NO. 3); CNPAGD(Y-OMe)RC-NH_2 (SEQ ID NO. 4); and CNP(Nle)GD(Y-OMe)RC-NH_2 (SEQ ID NO. 5).

In these embodiments of X_2 and X_3 both being a hydrophobic amino acid and X_4 being one to five amino acids, at least one of which is a positively charged amino acid directly adjacent to X_3, the compound encompassed by these formulae have a particular affinity for the $\alpha_{IIb}\beta_3$ integrin receptor. Unexpectedly, they have a higher affinity for $\alpha_{IIb}\beta_3$ at low calcium concentration, which is typical at the site of a clot as compared to $\alpha_{IIb}\beta_3$ at higher, physiological calcium concentration found elsewhere throughout the blood. Further characterization of these compounds is provided in Example II. Similarly, and also described in further detail in Example II, some of the compounds encompassed by the ensuing embodiments where X_2 is a positively charged amino acid and X_3 is a hydrophobic amino acid also have higher affinity for $\alpha_{IIb}\beta_3$ at low calcium concentration.

In an alternative embodiment of the invention, X_2 is a positively charged amino acid and X_3 is a hydrophobic amino acid. In another embodiment, X_2 is Arg, Lys, His, Orn or homo Arg; and X_3 is Ala, *t*-BuG,

Tic, Val, Ile, Leu, Phe, Tyr, Trp, a Tyr derivative, a Phe derivative, Nle, Nve, Cha, Nap, 2-Nal or Cit. In yet another embodiment, X_2 is Arg and X_3 is Phe, a Phe derivative, Tyr, a Tyr derivative or Trp. In a further embodiment, the compound selectively binds to $\alpha_{IIb}\beta_3$ and X_3 is a Tyr derivative and, more preferably, Tyr-OMe or Tyr-O-*n*-Bu. Preferred compounds falling within these formulae include CNPRGD(Y-OMe)RC-NH_2 (SEQ ID NO. 6); CNPRGD(Y-OMe)ACR (SEQ ID NO. 7); CNPRGD(Y-OMe)QC-NH_2 (SEQ ID NO. 8); CNPRGD(Y-OMe)ECR (SEQ ID NO. 9); and CNPRGD(Y-O-*n*-Bu)RC-NH_2 (SEQ ID NO. 10).

In yet a further embodiment of the present invention, X_2 is a positively charged amino acid; X_3 is either an amino acid that provides an ionic interaction with an integrin receptor or, alternatively, is an amino acid that provides a hydrogen bond with an integrin receptor; and X_4 is one to five amino acids, at least one of which is either a hydrophobic amino acid or a positively charged amino acid. In a preferred embodiment, X_2 is Arg, Lys, His, Orn or homoArg; X_3 is Asp, Glu, tetA, Msa, Tfsa, Psa, Asn, Ser, Thr or Tyr; and X_4 is at least one amino acid selected from Ala, tBuG, Tic, Pro, Val, Ile, Leu, Phe, Tyr, Trp, a Tyr derivative, a Phe derivative, Nle, Nve, Cha, Nap, 2-Nal, Cit, Arg, Lys, His, Orn, or homoArg. More preferably, X_2 is Arg; X_3 is Asp, Asn, Ser or Thr; and X_4 is at least one amino acid selected from tBuG, Phe, Tic, Pro or Arg.

Some of integrin-binding compounds of the above formulae corresponding to SEQ ID NO. 1 are selective for one or more integrin receptors, whereas others are not selective and bind multiple integrins. As used herein,

the terms "selective" and "selectively" mean that the integrin-binding compound used in the MRI contrast agent of the present invention preferentially binds to an integrin receptor with greater affinity than it binds to other integrins. It is recognized that a compound of the invention can specifically bind more than one integrin, yet still be considered selective because it does not non-specifically bind any integrin. The binding characteristics and selectively for one or more integrins can readily be determined, as described in Example II and other means well known in the art.

In one embodiment of the MRI contrast, the compound binds multiple integrins, but does not selectively bind any integrin. An example of a compound which binds multiple integrins, but not any one selectively, is (Pen)FARGDS(Tic)C-NH_2 (SEQ ID NO. 11).

In another embodiment of the invention, the compounds selectively bind multiple integrins, such G(Pen)RARGDNPCA (SEQ ID NO. 12); which selectively bind $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$, and (Pen)(Y-OMe)ARGDN(Tic)C-NH_2 (SEQ ID NO. 13) which selectively binds $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$.

The invention also encompases compounds which selectively bind to one integrin, such as those described above which are selective for $\alpha_{IIb}\beta_3$, as well as others. An example of other such compounds includes one which selectively binds to $\alpha_v\beta_3$ and has the structure (Mpa)RGDD(*t*-BuG)CK-NH_2 (SEQ ID NO. 14). As well, compounds preferably having X_3 as a small, aliphatic amino acid containing a hydroxyl or amide group can selectively bind to $\alpha_v\beta_5$. X_3 is preferalby Ser, Thr, Asn or Gln and specific examples are those compounds having the structures G(Pen)FRGDSFCA (SEQ ID NO. 15); GRGDTFEK-NH_2 (SEQ ID NO. 16); and G(Pen) FRGDNFCA (SEQ ID NO. 17).

The substituents X_1 and X_4, as described above, are, independently, zero to five amino acids. When present and between one and five amino acids, X_1 and X_4 can contain any naturally-occurring or non-naturally-occurring amino acids, including the D-form of the amino acids, amino acid derivatives and amino acid mimics, so long as the desired function and activity of the peptide is maintained.

Also as described above with respect to certain embodiments of the invention, X_1 and/or X_4 are one to five amino acids, wherein at least one amino acid is specified to meet a certain criteria. For example, as described above with respect to one embodiment, X_4 must have a positively charged amino acid directly adjacent to X_3. In yet another embodiment described above, X_4 can contain either a hydrophobic or a positively charged amino acid also adjacent to X_3.

With any of the above described embodiments, excepting when X_1 is absent, X_1 also preferably contains an amino acid capable of forming a bridge with another amino acid within the chemical compound. Preferably, the bridging amino acid is Cys, Pen, Mpa, Pmp, Pmc, Pas, Glu, Asp, Lys or Orn. Similarly for X_4, with any of the above described embodiments, excepting when X_4 is absent, X_4 also preferably contains an amino acid capable of forming a bridge with another amino acid within the chemical compound. Preferably, the bridging amino acid of X_4 is Cys, Pen, Mpa, Pmp, Pmc, Pas, Glu, Asp, Lys or Orn. Cyclization of the subject compounds through these and other amino acids is discussed in detail below.

The length of the integrin-binding compounds can vary, but generally they are small synthetic compounds on the order of about 14 amino acids or shorter. With those compassed encompassed by the formula of SEQ ID NO. 1, the compounds will of course vary in length depending on the substitutions for X_1 and X_4. The compounds must at least comprise X_2GDX_3 (SEQ ID NO. 18) and, therefore, are at least 4 amino acids, or the equivalent, in length. At the upper limit and based on the formula, the cyclic peptides can be as long as 14 amino acid residues. It should, however, be appreciated to those of skill in the art that the addition of one to a few amino acids to increase the peptide length beyond 14 residues could likely yield similar activity and, therefore, would not depart from the spirit and concept of the present invention. The length of the compounds will also depend on whether either or both X_1 and X_4 are present. For example, compounds encompassed within the present invention, include those of the formulas $Y-X_1-X_2-GD-X_3-Z$ (SEQ ID NO. 19) and $Y-X_2-GD-X_3-X_4-Z$ (SEQ ID NO. 20). The length of the compounds are preferably less than about 10 amino acids, and more preferably, less than about 7 amino acids in length.

The Y substituent in the above formula is an amino-terminal group. The term "amino-terminal group" as used herein refers to the amino group common to the amino terminus of a peptide (i.e., NH_2 and therfore Y is a hydrogen atom) or an amino group commonly employed to block or protect the amino functionality, for example, from enzymatic degradation. Examples of such amino-terminal groups used to block or protect the amino functionality include $COCH_3$, CO-alkyl, an alkyl group, CH_2Ph, COPh, $COOCH_2Ph$, COO-alkyl and CO-alkyl-NH_2. The

abbreviation "Ph" indicates a "phenyl" group (C_6H_5). The terms "alkyl" or "alkyl group" mean a C_1 to C_6 alkyl, including such radicals as methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, amyl, hexyl and the like.

The Z substituent is a "carboxy-terminal group" which refers to the carboxyl group common to the carboxy terminus of a peptide (i.e., COOH and therfore Z is a hydoxyl group) or a carboxy group commonly employed to block or protect the carboxy functionality, for example, from enzymatic degradation. Examples of such carboxy-terminal groups used to block or protect the carboxy functionality include NH_2, $NH\text{-}NH_2$, O-alkyl, SH, S-alkyl, N-alkyl, $NHCH_2Ph$ and $NH\text{-alkyl-}NH_2$. The abbreviation "Ph" and term "alkyl" are the same as defined above with reference to the Y substituent.

As indicated by these Y and Z substituents of the above generic formula, a compound of the MRI contrast agent can be modified at either or both the amino or carboxyl terminus. Such modifications should take into account tht there must a terminus or side chain of an amino acid capable of coupling to the chelator as described above. For example, Y is a hydrogen atom, the unprotected primary amine at the amino terminus can be used for coupling. Alternatively, amino-terminus can be protected, for example with and acetyl group, and the carboxy terminus can be modified such that a free primary amine is available for coupling to the chelator, as for example when Z is NH_2, $NH\text{-}NH_2$, $NH\text{-alkyl-}NH_2$. Alternatively, side chains of amino acids, such as the primary amine of Lys can be used in coupling the chelator to the compound.

Methods for modifying the termini are well known in the art. As described above, modifications for the amino terminus (Y) include, $COCH_3$ ("Ac"), CO-alkyl, an alkyl group, CH_2Ph, COPh, $COOCH_2Ph$, COO-alkyl and CO-alkyl-NH_2. Modifications for the carboxyl terminus (Z) include, NH_2, NH-NH_2, O-alkyl, SH, S-alkyl, N-alkyl, $NHCH_2Ph$ and NH-alkyl-NH_2.

To perform such modifications, peptides and peptidic-like compounds can be manipulated, for example, while still attached to a resin to obtain N-terminal modified agents such as an acetylated peptide or can be removed from the resin using hydrogen fluoride or an equivalent cleaving reagent and then modified. Compounds synthesized containing the C-terminal carboxyl group (Wang resin) can be modified after cleavage from the resin, or in some cases, prior to solution phase synthesis. Methods are well known in the art for acetylation, amidation and any of the other above-described modifications.

Any of the above described embodiments of X_1, X_2, X_3, X_4, Y and Z can be combined, in any combination, to arrive at yet further embodiments of the present invention than those specific combinations identified above and in the claims.

The integrin-binding compounds used in the subject MRI contrast agents can be synthesized by any of the suitable methods well known in the art including methods of chemical synthesis. For example, the linear counterpart can be synthesized using commercially available automated peptide synthesizers such as those manufactured by Applied Biosystems, Inc., Foster City, CA. The compounds can be synthesized using amino acids or amino acid analogs, the active groups of which are

protected as necessary using, for example a t-butyldicarbonate (t-Boc) group or a fluorenylmethoxy carbonyl (Fmoc) group. Amino acids and amino acid analogs can be purchased commercially (Sigma Chemical Co.; Advanced Chemtec) or synthesized using methods known in the art. Compounds synthesized using the solid phase method can be attached to resins, including 4-methylbenzhydrylamine (MBHA), 4-(oxymethyl)-phenylacetamido methyl and 4-(hydroxymethyl)phenoxymethyl -copoly(styrene-1% divinylbenzene) (Wang resin), all of which are commercially available, or using other resins well known in the art. The material so synthesized can be cleaved, precipitated and further purified, for example by high performance liquid chromatography (HPLC). Although a purity of greater than 95 percent for the synthesized product is generally desirable, lower purity may be acceptable. The synthesized compound is cyclized using methods well known in the art, as for example, those described below.

The integrin-binding chemical compound of the MRI contrast agent can be a cyclic compound resulting from a bridge between two amino acids within the compound. The cyclic bridge within a specifically described compound is identified herein by underlining (_____). Two amino acids, not including G or D, form the bridge. In other words, the G or D amino acids do not contribute to the chemical bond of the bridge. The compound, however, can be cyclized through a bridge between any two of the other amino acids present in a given integrin-binding compound of the MRI contrast agent, provided that the two amino acids are on opposite sides of the G-D sequence. Therefore, with reference to SEQ ID NO. 1 the compounds can be cyclized through (1) X_1 and X_4, (2) X_2 and X_4, (3) X_2 and X_3, and (4) X_3 and X_1. Preferably the cyclization is the result of bridging

between (1)) X_1 and X_4 or (2) X_2 and X_4, particularly when X_3 is a hydrophobic amino acid, an amino acid that provides an ionic interaction with an integrin receptor or when X_3 is an amino acid that provides a hydrogen bond with an integrin receptor. Regardless of which two amino acids form the bridge, there must be an amino acid side-chain or terminus of the compound available for coupling to the chelator as described above.

As used herein, the term "cyclic" refers to a compound having an intramolecular bond between the two amino acids forming a bridge within a given compound. The intramolecular bond includes backbone to backbone, side-chain to backbone, and side-chain to side-chain cyclizations. An example of a backbone to backbone cyclization is a bridge formed between the amino group of the amino terminus and the carboxylic acid of the carboxy terminus. Examples of a side-chain to side-chain cyclization include, for instance, disulfide bridges formed through cysteine residues or other sulfur containing amino acids capable of forming such bridges, or alternatively, a bridge formed between the side chain of a basic amino acid, such as Lys, and the side chain of an acidic amino acid, such as Glu.

More specifically, cyclization can be achieved where the compound contains two sulfur-containing amino acids, or other moieties through a disulfide bond. Examples of useful sulfur-containing moieties are Cys, Pen, Mpa, Pmp and Pmc. For example, a bridging structure can be created through a disulfide bond between Pmp and Cys (or similar structures). Where the residues contain sulfhydryls, a disulfide bridge can be formed by oxidizing a dilute aqueous solution of the peptides with $K_3[Fe(CN)_6]$.

Alternatively, cyclization can be accomplished through the formation of a peptide bond or alkyl bridge structures using, for example, Pas. For example, an alkyl bond between Pas and an amino acid methylene moiety (or similar structure) can be used for forming alkyl bridge structures.

The cyclized compounds of the present invention can also be prepared by forming a peptide bond between non-adjacent amino acid residues. As used herein, the term "peptide bond" or "peptide linkage" refers to an amide linkage between a carboxyl group of one amino acid and the α-amino group of another amino acid. Side-chain to side-chain cyclizations can be performed by using N^{α}-Boc-amino acids together with OFm/Fmoc side-chain protection for Asp and Lys residues as described by Felix et al., Int. J. Peptide Protein Res., 31:231 (1988). Alternatively, side-chain to backbone cyclizations can be performed using this procedure. Alternative amino acids useful for side-chain to side-chain or side-chain to backbone cyclizations are Glu and Orn. Backbone to backbone cyclizations can be performed in solution on side chain protected peptides. Alternative methods of making cyclized peptides are disclosed, for example, in WO 91/01331 entitled "SMALL CYCLIC PEPTIDE AGGREGATION INHIBITORS," published 7 February 1991. Bridges can also be formed where the amide bond cyclizes the peptide to form a lactam, as in the case of (Nle)GD(Y-OMe)REK-NH_2 (SEQ ID NO. 3). Lactam bridges can also be used for end-to-end cyclizations with compounds of the formula X_2-G-D-X_3 (SEQ ID NO. 21), such as for example, R-G-D-K (SEQ ID NO. 22).

Backbone to backbone cyclizations can be performed in solution on side chain protected peptides. Briefly, linear side chain protected peptides are

synthesized on *O*-chlorotrityl resin (Barlos et al., Tetrahedron Lett., 30:3947 (1989)), using N^{α}-Fmoc amino acids. After assembly of the sequence on the resin, the N^{α}-Fmoc is removed and the side chain protected peptide is cleaved from the *O*-chlorotrityl resin with acetic acid. After removal of the acetic acid by rotary evaporation, the peptide is cyclized in solution with the BOP reagent. The solvent is removed by rotary evaporation and the side chain protection then is removed by treatment with trifluoroacetic acid to provide the backbone to backbone cyclic compound.

Alternative methods of making cyclized compounds are disclosed, for example, in WO 91/01331 entitled "SMALL CYCLIC PEPTIDE AGGREGATION INHIBITORS," published 7 February 1991. As described therein, for example, Bromoacetyl-Gly-Arg(g-2,2,5,7,8-pentamethylchroman-ß-sulfonyl)-Gly-Asp(beta-t-butyl)-Cys(S-triphenylmethyl)-O-(polymer resin) can be prepared using standard solid phase peptide synthesis utilizing Fmoc protecting group chemistry on a p-alkoxybenzyl alcohol resin. Repeated treatment of the resin bound peptide with a 1% solution of trifluoroacetic acid in dichloromethane results in cleavage of the S-triphenylmethyl group as evidenced by the bright yellow of the solution. Treatment is continued until dissipation of the yellow color (ca. 1.5 L of the cleavage solution is required per gram of resin bound peptide). After complete cleavage of the S-triphenylmethyl group, the resin bound peptide is washed several times with a 5% solution of N-methylmorpholine in N,N-dimethylacetamide and then shaken in pure N,N-dimethylacetamide for 12 hours to complete the cyclization. Treatment of the cyclized resin bound peptide with trifluoroacetic acid containing (v/v) 1% phenol, 1% anisole and 1% ethanedithiol effects concomitant cleavage of the remaining protective groups

and cleavage of the desired product from the resin, which can then be purified, for example by HPLC using a 4.6 mm x 250 mm column containing 10 micron, 300 Angstrom pore size C-18 packing. The elution of the column was with an acetonitrile 0.1% aqueous trifluoroacetic acid gradient going from 0% - 40% acetonitrile linearly over 80 minutes.

Although the integrin-binding compound of the MRI contrast agent is described with reference to these specific compounds, it is understood that functional equivalents known to those skilled in the art can be substituted for the given sequence without departing from the spirit of the invention. Compounds having sequences other than those specifically identified herein are also included in the invention, provided that they exhibit the requisite functional criteria. However, as will be readily appreciated from the description, the scope of the compounds in the MRI contrast agents does not include proteins such as naturally-occurring antibodies.

In addition to binding integrin, the compounds of the used in the subject MRI agents have cell attachment activity. As used herein, the phrase "cell attachment activity" means that a compound has the functional property of being able to induce a cell to adhere or connect or bind to the compound, thereby forming contact between the compound and the cell. Such activity includes inhibition of platelet aggregation. Once the compounds are synthesized, they can readily be tested for such activity by standard assays which are routine to perform. For instance, JY cell attachment assays or platelet aggregation assays, such as those described in Example II, are useful for assessing cell attachment activity.

METALS

In the present invention, a paramagnetic metal enhances contrast during MRI. As is commonly defined, a "paramagnetic metal" is a metal ion with one or more unpaired electrons, which confer a magnetic moment. A variety of paramagnetic metals are known to useful for enhancing contrast during MRI, any one of which can be used with the present invention including, for example, Mn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Gd^{3+}, Eu^{3+}, Dy^{3+}, Pr^{3+}, Cr^{3+}, Co^{3+}, Fe^{3+}, Ti^{3+}, Nd^{3+}, Sm^{3+}, Y^{3+}, Tb^{3+}, Ho^{3+}, Er^{3+}, Pa^{4+} and V^{4+}.

The skilled artisan will readily select a metal according to dose required to detect a thrombus and the toxicity of the metal to a subject. The selection of the metal is discussed Tweedle et al., *supra*, pp. 796-797. In general, the desired dose for an individual metal will be proportional to its relaxivity, modified by the biodistribution, pharmacokinetics and metabolism of the metal. In one embodiment, the metal of the MRI agent is a trivalent cation, for example Gd^{3+}, Eu^{3+}, Dy^{3+}, Pr^{3+}, Cr^{3+}, Co^{3+}, Fe^{3+}, Ti^{3+}, Nd^{3+}, Sm^{3+}, Y^{3+}, Tb^{3+}, Ho^{3+} and Er^{3+}. A particularly useful metal to enhance MRI contrast is Gd^{3+}, which has a comparatively high relaxivity and low toxicity, combined with the further advantage that it exists in only one biologically accessible oxidation state, thereby reducing undesirable metabolism of the metal. Another particularly useful metal is Cr^{3+}, which is relatively inexpensive.

One advantage of the present invention compared to alternative detection methods is that the patient is not exposed to radiation. By using such nonradioactive isotopes in the imaging process, such as Gd^{3+} or others described above, exposing the patient to radiation can be avoided.

In preferred embodiments of the present invention, the MRI contrast agent comprises a Gd^{3+} paramagnetic metal, a DTPA chelator, and a compound having one of the following structures: (Nle)GD(Y-OMe)REK-NH_2 (SEQ ID NO. 3); CNPAGD(Y-OMe)RC-NH_2 (SEQ ID NO. 4); or CNP(Nle)GD(Y-OMe)RC-NH_2 (SEQ ID NO. 5), wherein the Gd^{3+} is complexed with DTPA and the compound is conjugated to DTPA.

In yet other preferred embodiments of the invention, the MRI contrast agent comprises a Gd^{3+} paramagnetic metal, a DTPA chelator, and a compound having one of the following structures: (Mpa)RGDD(*t*-BuG)CK-NH_2 (SEQ ID NO. 14); CNPRGD(Y-OMe)RC-NH_2 (SEQ ID NO. 6); CNPRGD(Y-O-n-Butyl)RC-NH_2 (SEQ ID NO. 10); G(Pen)FRGDSFCA (SEQ ID NO. 15); GRGDTFEK-NH_2 (SEQ ID NO. 16); G(Pen)RARGDNPCA (SEQ ID NO. 12); (Pen)(Y-OMe)ARGDN(Tic)C-NH_2 (SEQ ID NO. 13); or (Pen)FARGDS(Tic)C-NH_2 (SEQ ID NO. 11), wherein the Gd^{3+} is complexed with DTPA and the compound is conjugated to DTPA.

The present invention also provides a composition useful as a medicament comprising an MRI contrast agent and a physiologically acceptable carrier. The MRI contrast agents can be combined with a physiologically acceptable carrier to form a composition useful for the detection of thrombus. Physiologically acceptable carriers are well known in the art and include aqueous solutions such as physiologically buffered saline or other buffers or solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters. A physiologically acceptable carrier can contain acceptable compounds that act, for example, to stabilize the MRI contrast agent, increase the absorption of the agent, or extend the half-life in the circulation. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or

dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a physiologically acceptable carrier depends, for example, on the route of administration and on the particular physiochemical characteristic of the specific MRI contrast agent.

The present invention further provides a method of detecting thrombus in a subject. This method comprises administering to the subject an effective amount of a magnetic resonance imaging agent, and when the agent homes to a thrombus, detecting the thrombus using magnetic resonance imaging.

As used herein, "thrombus" means a clot formed from blood constituents and attached to a vessel within the cardiovascular system. The thrombus can be the result of any variety of pathologies or conditions, such as cardiovascular lesions, atherosclerotic plaques, vascular clots or thromboembolic events, such as myocardial infarction and other organ infarcts. The detection of thrombus can have veterinary uses. Accordingly, by "subject" is meant a human being or other vertebrates that are the object of research, treatment, diagnosis, or experimentation. The subject is preferably a human. As used herein, the "agent homes" to a thrombus when it proceeds or is drawn to the thrombus location, as for example, by binding to an integrin at the site of thrombi.

Magnetic resonance imaging involves detecting differences in the relaxation rates for protons in water molecules of a subject's body. When a magnetic field is applied to the protons, which have a magnetic moment, they reach an excited state, but revert to their original

state by a process termed "relaxation." MRI allows medical diagnosis by visualizing differences in proton relaxation times in the subject's body. The MRI contrast agent of the present invention enhances the visualization by increasing the rate of relaxation, thereby increasing the contrast between water molecules at a thrombus and water molecules elsewhere in the subject's body.

The MRI contrast agent must be present in sufficient amounts to enable detection by an external camera, using magnetic field strengths that are reasonably attainable and compatible with patient safety and instrumental design. The requirements for such agents are well known in the art for those agents that have their effect upon water molecules in the medium, and are disclosed, for example, in Pykett, Scientific American, 246:78 (1982) and Ruge et al., Am. J. Radiol., 141:1209 (1987). For example, the effectiveness of the MRI contrast agent is concentration-dependent, and there is normally an optimum concentration of the paramagnetic metal for maximum efficacy. The optimal concentration will vary with the particular MRI contrast agent used, the locus of imaging, the mode of imaging, and the composition of the carrier. These factors, and their relative importance are known in the art.

The method of administering the MRI contrast agent is preferably parenterally, meaning intravenously, intraarterially, intrathecally, interstitially or intracavitarily. For imaging cardiovascular thrombus, intravenous or intraarterial administration is preferred.

It is contemplated that a subject will receive a dosage of MRI contrast agent sufficient to enhance the MRI signal at the site of a thrombus anywhere from 20 to 500%, the amount being a function of the particular metal, compound and mode of administration. When the MRI

contrast agent homes to a thrombus, the thrombus is scanned with a conventional MRI camera to visualize the thrombus.

The following Examples are intended only to illustrate, but not limit, the invention.

EXAMPLE I

Synthesis of Integrin-Binding Compounds

This example provides methods of chemically synthesizing compounds that are capable of binding to an integrin. Such a compound was coupled to a chelator that complexes a paramagnetic metal to form an MRI contrast agent of the present invention.

A. Synthesis of cyclic compounds having disulfide bonds:

Integrin-binding compounds cyclized through disulfide bridges, including CNPAGD(Y-OMe)RC-NH_2 (SEQ ID NO. 4) and CNP(Nle)GD(Y-OMe)RC-NH_2 (SEQ ID NO. 5), were synthesized as follows. Compounds were synthesized by the solid-phase method on an automated synthesizer (Applied Biosystems, Inc. Model 431A) (see Stewart and Young, in Solid Phase Peptide Synthesis, 2nd ed. (Pierce Chemical Co., Rockford, IL, 1984). Compounds having a C-terminal amide were synthesized using *p*-methylbenzhydrylamine (pMBHA) resin. Compounds having a C-terminal acid can be synthesized using, for example, a chloromethylated resin. Compounds having an N-terminal acetyl group can be acetylated using a mixture of acetic anhydride (20 eq) and diisopropylethylamine (20 eq) in N-methylpyrrolidone.

N-terminal tertbutyloxycarbonyl (Boc) protection was used for all amino acids.

Dicyclohexylcarbodiimide and hydroxybenzyltriazole were used in the coupling reactions. The extent of the reactions was monitored by the standard ninhydrin test.

Following synthesis, the compounds were removed from the resin and deprotected by adding anhydrous HF (10 ml/g of resin-bound compound) containing anisole (1 ml/g) at 0°C for 60 min. The HF was removed by evaporation and the residue was washed with anhydrous ether. The crude compounds were extracted with water or 15% aqueous acetic acid and the aqueous fractions were combined and cyclized as described below.

The crude acyclic compound was dissolved in 0.1 M ammonium bicarbonate (0.5 mg/ml) and stirred uncovered. The course of the reaction was monitored by HPLC. After cyclization was complete (several hours to several days), the solution was filtered and the compounds were purified and characterized as described in Example I.C. below.

B. Synthesis of cyclic compounds having lactam bridges:

Cyclic compounds that have a lactam bridge, such as (Nle)GD(Y-OMe)REK-NH_2 (SEQ ID NO. 3), can be synthesized as outlined here. The protected compound resin was synthesized using the pMBHA resin. The lactam bridge was formed while the compound was still on the resin using the method described by Felix et al., Int. J. Pept. Prot. Res. 31:231 (1988) and by Felix et al., Int. J. Pept. Prot. Res. 32:441 (1988).

Essentially, the method of Felix et al. used N^{α}-Boc-amino acids in combination with 9-fluorenylmethyl ester (OFm) side-chain protection. Asp and Glu were introduced using Boc-Asp(OFm)-OH and Boc-Glu(OFm)-OH. After coupling the final amino acid, OFm protecting

groups were selectively removed by treating the compound resin with 50% piperidine in dimethylformamide for 1 hr. The compound resin was then washed three times with 40 ml dichloromethane and mixed with a 6-fold excess of BOP reagent (benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate) in the presence of an 8-fold excess of diisopropylethylamine for 5 hr. Coupling reactions were repeated until the resin gave a negative ninhydrin test.

After the cyclization reaction was complete, compounds were removed from the resin and deprotected using anhydrous HF (10 ml/g of resin-bound compound) containing anisole (1 ml/g) at 0°C for 60 min. The HF was removed by evaporation and the residue washed with anhydrous ether. The crude compounds were then extracted with water or 15% aqueous acetic acid and the aqueous fractions combined, lyophilized, purified and characterized as described in Example I.C. below.

C. Purification and characterization of the compounds:

The crude compounds were purified via preparative RP-HPLC on a C_{18} silica gel column (Waters Delta-Pak™, 15 μm, 300 Å, 47 x 300 mm) eluting with a linear acetonitrile gradient (0-30%) with a constant concentration of trifluoroacetic acid (TFA; 0.1%, v/v) over 30 min at a flow rate of 40 ml/min. The purified compounds were analyzed by analytical RP-HPLC using a C-18 column (Vydac™, 5 μm, 300 Å, 4.5 x 250 mm). The purified compounds were recovered by lyophilization of the HPLC fractions, and had a purity of at least 95%. For analytical HPLC, a binary solvent system was used (water containing 0.1% TFA, acetonitrile containing 0.1% TFA). The solvent programs used linear gradients as follows: (1) 10-45% acetonitrile over 35 min with a flow

rate of 1.5 ml/min and (2) 0-70% acetonitrile over 30 min with a flow rate of 1.5 ml/min.

In some cases, the compound can be adjusted to a neutral pH and potassium ferricyanide added to the TFA compound to minimize polymerization that may result from the presence of the reducing agent. The potassium ferricyanide was removed by ion exchange chromatography and the compounds were lyophilized. The presence of thiol-reducing agents, which indicated the need to add potassium ferricyanide, was detected using Ellman's test (Arch. Biochem. Biophys. 82: 70 (1959).

To confirm the correct amino acid sequences were synthesized, the compounds were characterized by fast atom bombardment mass spectroscopy and by amino acid analysis. Amino acid analysis was performed on a Pickering Labs-Trione amino acid analyzer that was equipped with a Spectra-Physics UV detector. Hydrolysis of the compound samples for amino acid analysis was performed on 1 mg samples with 1 ml constant boiling 6N HCl. Samples were degassed, sealed under vacuum and heated for 24 hr. at 110°C.

EXAMPLE II

Characterization of the Integrin-Binding Properties of the Compounds

This example provides methods for characterizing the integrin-binding properties of the compounds described in Example I, including such characteristics as selectivity, even at varying calcium concentration, as well as cell attachment activity.

A. Selective binding of compounds to one or more integrins:

Integrin-binding compounds can bind selectively to one or more integrins. To characterize the selectivity of such binding, the affinity of the compounds for integrins $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ were determined and compared. The binding affinity of a compound for an integrin was assessed by the ability of the compound to displace the binding of an integrin to its integrin-specific ligand, such as fibrinogen, fibronectin or vitronectin.

The affinity of a compound to an integrin was determined by using a competitive enzyme-linked immunosorbent assay (ELISA). In such an ELISA assay, an integrin ligand such as fibrinogen, fibronectin or vitronectin, was immobilized on a plate. The binding of solubilized integrin to the immobilized ligand was detected with an anti-integrin antibody (see, e.g., Newman et al., Blood, 65:227-232 (1985)) followed by a labeled secondary conjugate. When various concentrations of integrin-binding compounds were also present, the affinity of the compound for the integrin was determined from the reduced level of binding of solubilized integrin to the ligand. Such ELISA assays were performed as follows.

1. ELISA assay for affinity of a compound to $\alpha_5\beta_1$ (fibronectin-binding integrin):

Microtiter plates were coated with 110 μl/well of human fibronectin (2 μg/ml in TBS). The plates were washed three times with TBS-Tween buffer (TBS with 0.05% Tween 20). Fifty μl of $\alpha_5\beta_1$ in TBS (containing 20 mM octylglucoside, 2 mM $NmCl_2$) was added to each well.

Fifty μl of an integrin-binding compound in the same buffer was added in 10-fold serial dilutions. The plates were incubated for 3 hrs at room temperature, then washed with 200 μl of TBS-Tween buffer. One hundred μl of affinity-purified rabbit anti-$\alpha_5\beta_1$ antibody was added to the wells and the plates were incubated for an additional 2 hrs. The wells were washed twice with TBS-Tween and then with distilled water.

Affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (100 μl/well) was added and incubated for 16 hrs at room temperature. The following day, the plates were washed twice with TBS-Tween and then with distilled water. One hundred μl of substrate mixture (10 mg *o*-phenylenediamine in 25 ml 0.1 M citrate-phosphate buffer, pH 5.0, plus 6 μl of 30% H_2O_2 added just before use) was added to the plates and allowed to develop. The development process was stopped by adding 50 μl of 4N H_2SO_4 to each well. The optical density at 492 nm compared to 405 nm was recorded and IC_{50} values were determined.

2. ELISA assay for affinity of a compound to $\alpha_{IIb}\beta_3$ (fibrinogen-binding integrin):

Microtiter plates were coated with 100 μl/well of 10 μg/ml purified fibrinogen and allowed to stand overnight at 4°C. The plates were washed three times with TBS-Tween buffer (0.150 M NaCl, 0.05 M Tris, pH 7.4 at room temperature, 0.05% Tween-20). Then, 50 μl of integrin-binding compound was added in various dilutions along with 100 mg/ml $\alpha_{IIb}\beta_3$ purified from human platelets (in 20 mM octyl glucosamine, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 50mM Tris, pH 7.4), incubated on a plate shaker at room temperature for 4 hrs. The plates were then washed three times with TBS-Tween and 100 μl of a polyclonal or monoclonal antibody specific for $\alpha_{IIb}\beta_3$ (such

as 1 μg/ml AP3). After 1 hr incubation at room temperature on a plate shaker, the samples were washed three times with TBS-Tween.

One hundred μl of GAMHRP (horseradish peroxidase conjugate of goat anti-mouse IgG diluted to 1:10,000 in TBS-Tween) was then added, and the mixture was incubated 1 hr at room temperature on a plate shaker. Samples were then washed three times with TBS-Tween, and 100 μl OPD substrate was added (0.67 mg/ml *o*-phenylenediamine in 16 mM citric acid, 50 mM $NaHPO_4$, pH 5.0, plus 0.0125% H_2O_2 added just before use). The color developed within 3 to 20 mins and the reaction was stopped with 100 μl of 1M H_2SO_4. The optical density at 492 nm was recorded and IC_{50} values were determined.

3. ELISA assay for affinity of a compound to $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (vitronectin-binding integrins):

Microtiter plates were coated with 2 μg/ml human vitronectin dissolved in PBS (50 ml/well) and stored overnight at 4°C. The plates were washed twice with wash buffer (PBS, 0.05% Tween-20) and blocked by incubating for 1 hr with about 150 μl/well of assay buffer (1% BSA in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 1mM $CaCl_2$, 1mM $MnCl_2$).

The plates were then emptied and 20 μl/well of integrin-binding compound (prepared in various dilutions in assay buffer) was added to each well. Twenty-five μl of a 30 μg/ml solution of purified $\alpha_v\beta_3$ in assay buffer was added to the well and the reaction was incubated on a plate shaker for 1 hr. Meanwhile, a antibody mixture was prepared (1.5 μg/ml mouse monoclonal anti-$\alpha_v\beta_3$ antibody solution with a 1:6000 dilution of anti-mouse-Fc-HRP antibody conjugate) and incubated for 1 hr. The assay plates were washed 4 times with PBS-Tween, and 50 μl/well

of the antibody mixture was added. After a 1 hr incubation, the plate was then washed 4 times.

The color reaction was developed with 50 μl/well of OPD substrate was added (0.67 mg/ml o-phenylenediamine in 16 mM citric acid, 50 mM $NaHPO_4$, pH 5.0, plus 0.0125% H_2O_2 added just before use). The reaction was stopped with 50 μl 1M H_2SO_4. The optical density at 492 nm compared to 405 nm was recorded and IC_{50} values were determined.

ELISA assay for $\alpha_v\beta_5$ was performed by a substantially similar method, except using $\alpha_v\beta_5$ and anti-$\alpha_v\beta_5$ antibodies.

4. Selective affinities of compounds to one or more integrins as determined by competitive ELISA assays:

Table I compares the relative affinities of various compounds for the indicated integrins in competitive receptor binding assays. It should be noted that in the present example, certain integrin-binding compounds were assayed in the form of closely related analogs. For example, SEQ ID Nos. 6, 10, 13 and 11 were assayed in their acetylated form. Compounds designated SEQ ID Nos. 14 and 16 were assayed in the form of analogs omitting a lysine near the C-terminus. There is an expectation that the results from the compounds of the present invention would be the same and that the results presented are therefore indicative of the selective affinities of such compounds. Affinity was shown in terms of the concentration of each compound required to inhibit 50% of the binding of an integrin to its integrin-specific ligand (IC_{50}). Values of IC_{50} that were less than 50 nM indicate that the compound has a strong binding affinity to the integrin.

TABLE I

SEQ ID NO.	IC_{50} (nM) $\alpha_{IIb}\beta_3$	IC_{50} (nM) $\alpha_v\beta_3$	IC_{50} (nM) $\alpha_v\beta_5$	IC_{50} (nM) $\alpha_5\beta_1$
6	12	330	6,100	8,700
10	6	200	10,000	1,500
14	120	6	550	390
15	3,400	2,700	46	1,800
16	nd	63	2	350
12	30	52	330	2
13	190	2	6	5
11	15	4	6	6

"nd" means the affinity was not determined

As can be seen from Table I, certain compounds were selective to a single integrin, while others were selective for more than one integrin. For example, SEQ ID NO. 15 selectively binds to only one integrin assayed, $\alpha_v\beta_5$. On the other hand, SEQ ID NO. 12 selectively binds to more than more integrin, namely $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$.

B. Binding of compounds and structural analogs thereof to integrins at different calcium concentrations:

The affinity of integrin-binding compounds can be cation-dependent. To characterize the effect of cation concentration on the ability of compounds to bind integrins, competitive ELISA assays were performed as described in Example II.A. In such assays, the concentration of calcium in different reactions was varied from 0.01 mM to 100 mM Ca^{2+}, and the ability of compounds to inhibit the binding of fibrinogen to $\alpha_{IIb}\beta_3$ purified was determined.

Table II compares the relative affinities of various compounds for $\alpha_{IIb}\beta_3$ in the presence of different calcium concentrations. It should be noted that compounds designated SEQ ID Nos. 6 and 8 were assayed in their acetylated form; nevertheless, the results presented are indicative of the calcium-dependence of the integrin-binding compounds comprised by the present invention. The affinities are presented in terms of the fold difference in IC_{50} when compared to a base value of the affinity in the presence of 0.01 mM Ca^{2+}.

TABLE II

SEQ ID NO.	amino acid sequence of integrin-binding compound	0.01 mM Ca^{2+}	0.1 mM Ca^{2+}	1 mM Ca^{2+}	10 mM Ca^{2+}	100 mM Ca^{2+}
6	CNPRGD(Y-OMe)RC-NH_2	1	2	7	65	221
7	RCNPRGD(Y-OMe)ACR	1	3	9	54	53
8	CNPRGD(Y-OMe)OC-NH_2	1	3	7	13	8
9	RCNPRGD(Y-OMe)ECR	1	2	2	3	5

It can be seen from these results that certain compounds, such as SEQ ID NOs. 6 and 7, have affinities that were highly calcium-dependent, while other analogs, such as SEQ ID NO. 9, were relatively calcium-independent.

C. Cell Attachment Activity

Routine assays, such as JY cell attachment assays or platelet aggregation assays are useful for assessing cell attachment activity of integrin-binding compounds.

1. JY cell attachment assay

JY cells are human-derived B lymphocyte cells that express the $\alpha_v\beta_3$ VnR but do not express the $\alpha_v\beta_5$ VnR. Integrin-binding compounds can analyzed for their ability to inhibit the attachment of JY cells to vitronectin-coated plates. In addition, the JY attachment assay, when compared with the receptor-specific enzyme-linked immunosorbent assays (ELISAs) described above, demonstrates the selectivity of RGD peptides for inhibiting binding due to the $\alpha_v\beta_3$ VnR.

JY cells are grown in suspension in RPMI media (Irvine Scientific; Irvine, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 200 mM glutamine and containing 100 IU/ml penicillin and 100 μg/ml streptomycin. A 96 well ELISA plate (Linbro, Titertek) is coated with 100 μl/well of 5μg/ml human Vn in sodium carbonate (pH 9.5) and incubated overnight at 4°C. The plate is rinsed 3x with PBS and blocked by adding 100 μl/well of 2 mg/ml BSA in PBS and incubating for 1 hr. Following blocking, the wells are washed 2x with PBS.

While the wells are being blocked, the JY cells are rinsed 2x with PBS, then resuspended to 10 x 10^5 cells/ml in serum-free RPMI containing 2 mg/ml BSA, 200 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.5 mM $MnCl_2$. At this time, 50 ng/ml phorbol myristate acetate (PMA; Sigma) is added to the cell suspension. PMA stimulates the cells and allows them to bind Vn.

Integrin-binding compounds are diluted in the serum-free RPMI and 50 μl of each dilution is added to duplicate wells. Fifty μl of the JY cell suspension is added to each well and the plate incubated at 37°C for 45

min, 7% CO_2. Nonadherent cells are gently aspirated from the wells and the wells rinsed 1x with PBS. Cells are fixed in 10% formalin in PBS for 10 min, then stained with 0.1% toluidine blue for 1 hr. The plate is rinsed with distilled water and the cells solubilized using 1% SDS. The amount of staining is determined by spectrophotometry at 595 nm and the IC50 calculated as the concentration of an integrin-binding compound that inhibited JY cell binding to Vn by 50%.

2. Platelet aggregation

Platelet aggregation assays also provide an indicia of an integrin-binding compound's cell attachment activity. This example provides platelet aggregation studies for various peptides of the present invention. The studies compare the effect of the peptide on aggregation in both citrated and heparinized plasma. Because citrate is a chelating agent for calcium ions, the citrated plasma has a low calcium concentration as is characteristic of a clot site. In contrast, heparinized plasma has a calcium concentration similar to that of physiological conditions elsewhere in the blood stream.

Whole blood was drawn by a licensed phlebotomist from the antecubital vein using a 21 gauge butterfly infusion set and syringe from normal human volunteers. Platelet-rich plasma (PRP) was prepared by centrifuging heparinized (20 Units lithium porcine mucosa heparin/ml whole blood) or citrated (0.38% sodium citrate) whole blood for 12 min. at 200 relative centrifugal force (RCF). Platelet-poor plasma (PPP) is prepared by centrifuging PRP at 750 RCF for 12 min. and collecting the supernatant. Blood cell counts were performed using a Serano Baker automated cell counter. PRP platelet counts were adjusted to 200,000/μl by dilution with PPP. Final plasma free Ca^{+2} concentrations

were approximately 0.18 mM for citrated plasma and 2.3 mM for heparinized plasma. Platelet aggregation was determined spectrophotometrically utilizing a four channel aggregometer (Chrono-Log 570) by measuring the increase of light transmission through a stirred suspension of PRP maintained at 37°C. Dilutions of the peptides were added to the suspensions and aggregation induced with 10 μM adenosine diphosphate (ADP). Values were expressed as percentage of aggregation in the absence of peptides. This represents the percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission respectively.

Peptide anti-aggregation potencies were determined from dose-responsive curves for the inhibition of the maximum aggregation responses stimulated by high doses of ADP (10 μm). The 50% inhibitory concentration of each peptide (IC_{50}) was determined by regression analysis of these curves.

Peptides of the present invention which were tested using the above procedures include CNPAGD(Y-OMe)RC-NH_2 (SEQ ID NO. 4), CNP(Nle)GD(Y-OMe)RC-NH_2 (SEQ ID NO. 5) and CNPRGD (Y-OMe) RC-NH_2 (SEQ ID NO. 6). It should be noted that compounds designated SEQ ID Nos. 4, 5 and 6 were assayed in their acetylated form; however, the results presented are indicative of the effect on plate aggregation from the integrin-binding compounds comprised by the present invention.

The results of these experiments are provided in Table III. As shown in the Table, each of the peptides tested have inhibitory effects on platelet aggregation stimulated with ADP. The 50% inhibitory concentration of each peptide (IC_{50}) is provided in Table III.

TABLE III		
PLATELET AGGREGATION IN CITRATED PLASMA		
SEQ ID NO.	SEQUENCE	IC_{50} (μg/ml)
4	CNPAGD(Y-OMe)RC-NH_2	309
5	CNP(Nle)GD(Y-OMe)RC-NH_2	43
6	CNPRGD(Y-OMe)RC-NH_2	0.2

The data provided herein evidences that the subject peptides are useful antithrombotic agents. Importantly, peptides of the present invention have a higher apparent affinity for GP-IIb/IIIa at low calcium concentration as compared to GP-IIb/IIIa at higher calcium concentration. Peptides of the present invention would inhibit platelet aggregation only at the site of a clot and not throughout the whole blood stream.

EXAMPLE III

Synthesis of the MRI Contrast Agent

This example provides a general method for synthesizing the MRI contrast agent of the present invention by first coupling a chelator to an integrin-binding compound described in Examples I and II, and then complexing a paramagnetic metal to the chelator. The resulting MRI contrast agent can be analyzed by spectral and HPLC methods.

In the first step, a chelator was covalently linked to an integrin-binding compound. When using diethylenetriaminepentaacetic acid (DTPA), a chelator of trivalent cationic metals, the dianhydride of DTPA can covalently link the DTPA to a primary amine of a compound (see Hnatowich et al., Science 220: 613-615 (1983). DTPA

anhydride, which is commercially available from Sigma Chemical Co., St. Louis, MO, was suspended in dry chloroform. An aliquot of the slurry was placed in a glass reaction vial and the chloroform was blown off with nitrogen. An aliquot of the integrin-binding compound was solubilized in HBS (50 mM HEPES, pH 7.0, saline) and added to the dry DTPA anhydride at a 0.9:1 molar ratio (DTPA:compound). The binding of DTPA to the compound was virtually instantaneous.

In the next step, a paramagenetic metal was complexed with the chelator. A solution of $GdCl_2$ (1 M in water) was then added to the DTPA-compound solution in a 2:1 ratio (Gd:compound). The reacted solution was applied to an Alltech "Maxi-Clean"™ C_{18} cartridge with a 1 ml bed volume (preactivated with methanol and washed with water and HBS, pH 6.75). The column was washed with 5 ml pH 6.75 HBS, then the product was eluted with 50% acetonitrile HBS.

The resulting product was analyzed by spectroscopy. DTPA exhibits an absorbance peak at 215 nm. The integrin-binding compound has absorbance peaks at 220 nm and 280 nm. By observing the optical density at 215 nm and 280 nm, the elution positions of DTPA and compound can be differentiated.

The resulting product was also analyzed by HPLC on a Vydac™ Type 214TP54 C4 protein column. Two hundred μl samples of a 1:100 dilution of the product were loaded on the column with a flow rate of 1 ml/min using the following gradient: 0 min to 10 min at 5% acetonitrile, 5 min to 50 min in a linear gradient from 10% to 50% acetonitrile, 50 min to 52 min in a linear gradient from 50% to 80% acetonitrile, 52 min to 54 at 80% acetonitrile, 54 min to 56 min in a linear gradient from 80% to 5% acetonitrile, and 56 min to 70 min at 5%

acetonitrile. The uncoupled compound eluted at 17.9 minutes and the product eluted at 21 minutes.

The stability of the product was monitored by RP-HPLC. The product was tested for stability by mixing with, and incubated in, whole blood, plasma, serum, and water at 4°c, 20°c and 37°C. Samples at various time points were taken over a 36-hour period and diluted and injected on an analytical HPLC with appropriate reverse phase columns as described above. Quantification of the product's stability was performed by comparing the peak area integration of the product sample with standard concentrations of the integrin-binding compound and DTPA. Unmodified integrin-binding compounds have been previously demonstrated to be very stable in whole blood *in vitro* and *in vivo* in comprehensive stability and pharmacokinetic studies. (Nishit et al., J. Cardiovasc. Pharmacol., 25:888-897 (1995)).

EXAMPLE IV

In Vitro Human Blood Clot Assay for Screening Integrin-Binding Compounds and MRI Imaging Agents

This example provides a method for screening candidate agents for affinity to human blood clots. Such agents can be, for example, an integrin-binding compound or an MRI imaging agent.

Whole blood is drawn from human subjects from the antecubital vein using a 21 gauge butterfly infusion set and syringe. Coagulation of the blood is prevented by gently mixing the blood with one tenth volume of 0.129 M sodium citrate in a sterile polypropylene culture tube. Recalcification, contact with glass, and addition of thrombin are all methods used to induce coagulation and clot formation. Routinely, 100 μl aliquots of the citrated blood are mixed with 10 μl of 10 U/ml thrombin

in 1.5 ml polypropylene microtubes. The thrombi are allowed to form and contract for 2 hrs at room temperature.

Experiments can be performed with preformed clots to simulate the effect on an established, aged clot. Preformed clots are washed with buffer and incubated with the candidate agents in buffer, or alternatively blood or plasma. One hour of incubation of 100 μl of a candidate agent was sufficient for saturable binding.

An alternative method for doing an initial *in vitro* screen is to purify the DTPA-compound over the "Maxi-Clean"™ C_{18} cartridge, then add the paramagnetic metal, for example ^{51}Cr on a 1:1 molar ratio. Free metal is then removed from the product by size-exclusion or cation-exchange chromatography. The radioactivity that is incorporated in the clot can be determined with a gamma-counter.

Experiments also can be performed in the presence of the compound or MRI imaging agent while the thrombi are forming, thus simulating an actively forming thrombus. In such experiments, the same protcol is followed, except that the agent is added to the blood prior to adding the thrombin. After clots are allowed to form and contract for 2 hrs as above, they are washed and counted. Unlike the experiments performed using preformed clots, experiments involving actively forming thrombi are always incubated in whole blood, not plasma or buffer.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the inventions. Accordingly the invention is limited only by the claims.

WE CLAIM:

1. A magnetic resonance imaging (MRI) contrast agent comprising an integrin-binding compound which binds to the RGD site of an integrin, said compound being coupled to a chelator capable of complexing a paramagnetic metal.

2. The MRI contrast agent of claim 1, wherein said compound is X-G-D-containing (SEQ ID NO. 23) and further wherein X is selected from the group consisting of a positively charged amino acid and a hydrophobic amino acid.

3. The MRI contrast agent of claim 2, wherein the X-G-D-containing compound is linear.

4. The MRI contrast agent of claim 2, wherein the X-G-D-containing compound is cyclic.

5. An MRI contrast agent, comprising:

a paramagnetic metal,

a chelator, wherein the chelator complexes the metal; and

a chemical compound which is capable of binding with an integrin and has the structure:

$Y-X_1-X_2-G-D-X_3-X_4-Z$ (SEQ ID NO. 1), wherein

two amino acids, excluding G and D form a bridge;

Y an amino-terminal group;

X_1 is zero to five amino acids;

X_2 is selected from the group consisting of a hydrophobic amino acid and a positively charged amino acid;

X_3 is selected from the group consisting of hydrophobic amino acid, an amino acid that provides an ionic interaction with an integrin receptor, and an amino acid that provides a hydrogen bond with an integrin receptor;

X_4 is zero to five amino acids; and

Z is a carboxy-terminal group;

provided that the compound is coupled to the chelator and the compound has cell attachment activity and further provided that the bridge is between a pair of amino acids selected from the group consisting of (1) X_1 and X_4 , (2) X_2 and X_4, (3) X_2 and X_3 and (4) X_3 and X_1.

6. The MRI contrast agent of claim 5, wherein:

X_2 and X_3 are, independently, a hydrophobic amino acid;

X_4 is one to five amino acids, at least one of which is a positively charged amino acid directly adjacent to X_3; and

provided that the bridge is between a pair of amino acids selected from the group consisting of (1) X_1 and X_4 and (2) X_2 and X_4.

7. The MRI contrast agent of claim 6, wherein:

X_2 and X_3 are, independently, selected from the group consisting of Ala, *t*-BuG, Tic, Val, Ile, Leu, Phe, Tyr, Trp, a Tyr derivative, a Phe derivative, Nle, Nve, Cha, Nap, 2-Nal and Cit; and

X_4 is one to five amino acids, at least one of which is a positively charged amino acid directly adjacent to X_3, wherein said positively charged amino acid is selected from the group consisting of Arg, Lys, His, Orn and homoArg.

8. The MRI contrast agent of claim 7, wherein:

X_2 is selected from the group consisting of Ala, Val, Ile, Leu, Phe, Tyr, Trp, Nve, Nle, Cha and Nap;

X_3 is selected from the group consisting of Phe, a Phe derivative, Tyr, a Tyr derivative and Trp; and

X_4 is at least an Arg amino acid residue, said residue being directly adjacent to X_3.

9. The MRI contrast agent of claim 8, wherein X_2 is selected from the group consisting of Ala, Leu and Nle and X_3 is Tyr-OMe.

10. The MRI contrast agent of claim 6, wherein the compound has one of the following structures:

(Nle)GD(Y-OMe)REK-NH_2 (SEQ ID NO. 3);

CNPAGD(Y-OMe)RC-NH_2 (SEQ ID NO. 4); and

CNP(Nle)GD(Y-OMe)RC-NH_2 (SEQ ID NO. 5).

11. The MRI contrast agent of claim 5, wherein:

X_2 is a positively charged amino acid;

X_3 is a hydrophobic amino acid; and

provided that the bridge is between a pair of amino acids selected from the group consisting of (1) X_1 and X_4 and (2) X_2 and X_4.

12. The MRI contrast agent of claim 11, wherein:

X_2 is selected from the group consisting of Arg, Lys, His, Orn and homo Arg; and

X_3 is selected from the group consisting of Ala, *t*-BuG, Tic, Val, Ile, Leu, Phe, Tyr, Trp, a Tyr derivative, a Phe derivative, Nle, Nve, Cha, Nap, 2-Nal and Cit.

13. The MRI contrast agent of claim 12, wherein:

X_2 is Arg; and

X_3 is selected from the group consisting of Phe, a Phe derivative, Tyr, a Tyr derivative and Trp.

14. The MRI contrast agent of claim 13, wherein the compound selectively binds to $\alpha_{IIb}\beta_3$ and X_3 is a Tyr derivative.

15. The MRI contrast agent of claim 14, wherein the Tyr derivative is selected from the group consisting of Tyr-OMe and Tyr-O-*n*-Bu.

16. The MRI contrasting agent of claim 15, wherein the compound has one of the following structures:

CNPRGD(Y-OMe)RC-NH_2 (SEQ ID NO. 6);

CNPRGD(Y-OMe)ACR (SEQ ID NO. 7);

CNPRGD(Y-OMe)QC-NH_2 (SEQ ID NO. 8);

CNPRGD(Y-OMe)ECR (SEQ ID NO. 9); and

CNPRGD(Y-O-*n*-Bu)RC-NH_2 (SEQ ID NO. 10).

17. The MRI contrast agent of claim 5, wherein:

X_2 is a positively charged amino acid;

X_3 is selected from the group consisting of an amino acid that provides an ionic interaction with an integrin receptor and an amino acid that provides a hydrogen bond with an integrin receptor;

X_4 is one to five amino acids, at least one of which is selected from the group consisting of a hydrophobic amino acid directly adjacent to X_3 and a positively charged amino acid directly adjacent to X_3; and

provided that the bridge is between a pair of amino acids selected from the group consisting of (1) X_1 and X_4 and (2) X_2 and X_4.

18. The MRI contrast agent of claim 17, wherein

X_2 is selected from the group consisting of Arg, Lys, His, Orn and homoArg;

X_3 is selected from the group consisting of Asp, Glu, tetA, Msa, Tfsa, Psa, Asn, Ser, Thr and Tyr; and

X_4 is at least one amino acid selected from the group consisting of Ala, tBuG, Tic, Pro, Val, Ile, Leu, Phe, Tyr, Trp, a Tyr derivative, a Phe derivative, Nle, Nve, Cha, Nap, 2-Nal, Cit,

Arg, Lys, His, Orn, and homoArg, said amino acid being directly adjacent to X_3.

19. The MRI contrast agent of claim 18, wherein

X_2 is Arg;

X_3 is selected from the group consisting of Asp, Asn, Ser and Thr; and

X_4 is at least one amino acid selected from the group consisting of tBuG, Phe, Tic, Pro and Arg, said amino acid being directly adjacent to X_3.

20. The MRI contrast agent of claim 17, wherein the compound binds multiple integrins, but does not selectively bind any integrin.

21. The MRI contrast agent of claim 20, wherein the compound has the structure

(Pen)FARGDS(Tic)C-NH_2 (SEQ ID NO. 11).

22. The MRI contrast agent of claim 17, wherein the compound selectively binds multiple integrins.

23. The MRI contrast agent of claim 22, wherein the compound has one of the following structures:

G(Pen)RARGDNPCA (SEQ ID NO. 12); and

(Pen)(Y-OMe)ARGDN(Tic)C-NH_2 (SEQ ID NO. 13).

24. The MRI contrast agent of claim 17, wherein the compound selectively binds to one integrin.

25. The MRI contrast agent of claim 24, wherein the compound selectively binds to $\alpha_v\beta_3$ and has the structure (Mpa)RGDD(*t*-BuG)CK-NH_2 (SEQ ID NO. 14).

26. The MRI contrast agent of claim 24, wherein the compound selectively binds to $\alpha_v\beta_5$ and X_3 is a small, aliphatic amino acid containing a hydroxyl or amide group

27. The MRI contrast agent of claim 26, wherein X_3, is an amino acid selected from the group consisting of Ser, Thr, Asn and Gln.

28. The MRI contrast agent of claim 27, wherein the compound has one of the following structures:

G(Pen)FRGDSFCA (SEQ ID NO. 15);

GRGDTFEK-NH_2 (SEQ ID NO. 16); and

G(Pen)FRGDNFCA (SEQ ID NO. 17).

29. The MRI contrast agent of claim 5, wherein X_1 is one to five amino acids at least one of which is capable of forming a bridge with another amino acid within the chemical compound.

30. The MRI contrast agent of claim 24, wherein X_1 comprises at least one amino acid selected from the group consisting of Cys, Pen, Mpa, Pmp, Pmc, Pas, Glu, Asp, Lys and Orn.

31. The MRI contrast agent of claim 5, wherein X_4 is one to five amino acids, at least one of which is capable of forming a bridge with another amino acid within the chemical compound.

32. The MRI contrast agent of claim 31, wherein X_4 is one to five amino acids, at least one of which is selected from the group consisting of Cys, Pen, Mpa, Pmp, Pmc, Pas, Glu, Asp, Lys and Orn.

33. The MRI contrast agent of claim 5, wherein the compound is coupled to the chelator through any one substituent selected from the group consisting of Y, X_1, X_4 and Z.

34. The MRI contrast agent of claim 33, wherein the compound is coupled through Y or Z.

35. The MRI contrast agent of claim 5, wherein Y is selected from the group consisting of a hydrogen atom, $COCH_3$, CO-alkyl, an alkyl group, CH_2Ph, COPh, $COOCH_2Ph$, COO-alkyl and CO-alkyl-NH_2.

36. The MRI contrast agent of claim 5, wherein Z is selected from the group consisting of selected from the group consisting of OH, NH_2, NH-NH_2, O-alkyl, SH, S-alkyl, N-alkyl, $NHCH_2Ph$ and NH-alkyl-NH_2.

37. The MRI contrast agent of claim 5, wherein the metal is selected from the group consisting of Mn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Gd^{3+}, Eu^{3+}, Dy^{3+}, Pr^{3+}, Cr^{3+}, Co^{3+}, Fe^{3+}, Ti^{3+}, Nd^{3+}, Sm^{3+}, Y^{3+}, Tb^{3+}, Ho^{3+}, Er^{3+}, Pa^{4+} and V^{4+}.

38. The MRI contrast agent of claim 37, wherein the metal is a trivalent cation.

39. The MRI contrast agent of claim 38, wherein the metal is Gd^{3+}.

40. The MRI contrast agent of claim 38, wherein the metal is Cr^{3+}.

41. The MRI contrast agent of claim 37, wherein the isotope of the metal is nonradioactive.

42. The MRI contrast agent of any one of claims 1 or 5, wherein the chelator is selected from the group consisting of diethylenetriaminepentaacetic acid (DTPA), 1,4,7,10-tetraazacyclododecane (DOTA), ethylenediaminetetraacetic acid (EDTA) and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA).

43. The MRI contrast agent of claim 42, wherein the chelator is DTPA.

44. An MRI contrast agent, comprising a Gd^{3+} paramagnetic metal, a DTPA chelator, and a compound having one of the following structures:

(Nle)GD(Y-OMe)REK-NH_2 (SEQ ID NO. 3);

CNPAGD(Y-OMe)RC-NH_2 (SEQ ID NO. 4); and

CNP(Nle)GD(Y-OMe)RC-NH_2 (SEQ ID NO. 5),

wherein the Gd^{3+} is complexed with DTPA and the compound is conjugated to DTPA.

45. An MRI contrast agent, comprising a Gd^{3+} paramagnetic metal, a DTPA chelator, and a compound having one of the following structures:

(Mpa)RGDD(t-BuG)CK-NH_2 (SEQ ID NO. 14);

CNPRGD(Y-OMe)RC-NH_2 (SEQ ID NO. 6);

CNPRGD(Y-O-n-Butyl)RC-NH_2 (SEQ ID NO. 10);

G(Pen)FRGDSFCA (SEQ ID NO. 15);

GRGDTFEK-NH_2 (SEQ ID NO. 16);

G(Pen)RARGDNPCA (SEQ ID NO. 12);

(Pen)(Y-OMe)ARGDN(Tic)C-NH_2 (SEQ ID NO. 13); and

(Pen)FARGDS(Tic)C-NH_2 (SEQ ID NO. 11),

wherein the Gd^{3+} is complexed with DTPA and the compound is conjugated to DTPA.

46. A composition of matter comprising the MRI contrast agent of any one of claims 1 or 5, and a physiologically acceptable carrier.

47. A composition of matter comprising the MRI contrast agent of any one of claims 44 or 45 and a physiologically acceptable carrier.

48. A method of detecting thrombus in a subject, comprising administering to the subject an effective amount of the MRI contrast agent of

any one of claims 1 or 5, and when the agent homes to a thrombus, detecting the thrombus using magnetic resonance imaging.

49. A method of detecting thrombus in a subject, comprising administering to the subject an effective amount of the MRI contrast agent of any one of claims 44 or 45, and when the agent homes to a thrombus, detecting the thrombus using magnetic resonance imaging.

50. The method of claim 48 or 49, wherein the subject is a human.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18412

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 49/00; G01N 31/00, 33/48
US CL :424/9.34
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.11, 1.65, 1.69, 9.1, 9.3, 9.323, 9.34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,988,496 A (SRINIVASAN et al) 29 January 1991, see entire document, especially, column 1, lines 17-68; column 7, lines 55-67, column 8, lines 30-68.	1-50
Y	US 4,879,237 A (RUDSLAHTI et al) 07 November 1989, see entire document.	1-50
Y	CRAIG et al. Concept and Progress in the Development of RGD-Containing Peptide Pharmaceuticals. Biopolymers (Peptide Science). 1995, Vol. 37, No. 2, pages 157-175, especially, page 157, Introduction, pages 159-162, Design of the Pharmacophore.	1-50

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
02 DECEMBER 1997

Date of mailing of the international search report
14 JAN 1998

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/18412

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHENG et al. Design and Synthesis of Novel Cyclic RGD-Containing Peptides as Highly Potent and Selective Integrin $\alpha_{IIb}\beta_3$ Antagonist. Journal of Medicinal Chemistry. 07 January 1994, Vol. 37, No. 1, pages 1-8, see entire document.	1-50
Y	CHENG et al. 'Design & Synthesis of novel cyclic RGD peptides as highly potent & selective GPIIb/IIIa antagonists', In: Peptides: Chemistry, Structure, and Biology, Proceedings of the Thirteenth American Peptide Symposium. Edited by R. S. June 1993, pages 384-386, see entire document.	1-50
Y, P	US 5,659,041 A (POLLAK et al) 19 August 1997, see entire document, especially, column 5, lines 7-13.	1-50